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van Oijen, Antoine M.

Published in:
Current Opinion in Biotechnology

DOI:
10.1016/j.copbio.2010.10.002

Publication date:
2011

Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Biotechnology

Single-molecule approaches to characterizing kinetics of biomolecular interactions

Antoine M van Oijen

Single-molecule fluorescence techniques have emerged as powerful tools to study biological processes at the molecular level. This review describes the application of these methods to the characterization of the kinetics of interaction between biomolecules. A large number of single-molecule assays have been developed that visualize association and dissociation kinetics *in vitro* by fluorescently labeling binding partners and observing their interactions over time. Even though recent progress has been significant, there are certain limitations to this approach. To allow the observation of individual, fluorescently labeled molecules requires low, nanomolar concentrations. I will discuss how such concentration requirements in single-molecule experiments limit their applicability to investigate intermolecular interactions and how recent technical advances deal with this issue.

Address
Zernike Institute for Advanced Materials, Centre for Synthetic Biology, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Corresponding author: van Oijen, Antoine M (a.m.van.oijen@rug.nl)

Current Opinion in Biotechnology 2011, **22**:75–80

This review comes from a themed issue on
Analytical biotechnology
Edited by Matthias Heinemann and Uwe Sauer

Available online 29th October 2010

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2010.10.002

Introduction

The ability to observe biochemical reactions at the level of a single molecule has greatly contributed to our understanding of the molecular mechanisms that define life [1–11]. A major strength of studying processes at the level of individual molecules lies in the direct measurement of distributions of molecular properties, rather than their ensemble averages. By constructing histograms of particular molecular observables for many individual molecules, deviant subpopulations can be identified and characterized. Moreover, the recording of single-molecule trajectories allows us to follow molecular processes in real time and observe rare and short-lived intermediates. The absence of a need for synchronization of the entire ensemble of molecules allows us to extract detailed dynamical information from single-molecule trajectories, otherwise obscured in kinetic ensemble studies by dephasing processes.

Binding is arguably the most fundamental of biomolecular interactions, and as such has been investigated using a wide array of methods. Canonical kinetic methods are often limited to nonequilibrium measurements, are restricted in the rate and K_d ranges they can access, and provide only ensemble-averaged molecular information. Single-molecule tools on the other hand are ideally suited for the quantitative characterization of bimolecular association and dissociation kinetics under equilibrium conditions. Single-molecule methods do not only enable the direct observation of binding and unbinding events for a very precise determination of rate constants at a large range of rates and K_d's, but also allow the visualization of various types of heterogeneity in binding properties. Different members of a seemingly homogeneous collection of molecules may exhibit different binding kinetics (so-called static disorder), or individual members of a population may exhibit binding kinetics that change over time (dynamic disorder) [11–16]. Characterizing these different phenomena and correlating them with information on structural conformation will increase our understanding of the relationship between activity and structure, an important factor in both binding and enzymatic activity [14,17–22].

In this review, I will briefly discuss the methods available to measure biomolecular interaction kinetics, such as small molecule–protein, protein–protein, and protein–DNA interactions, at the single-molecule level and give examples of applications of these tools to *in vitro* problems. I will also address current limitations of these single-molecule approaches and possible strategies to overcome them.

Strategies to visualize binding kinetics

The visualization of the association and dissociation kinetics of a molecule with its binding partner is essentially a binary problem — only the bound and unbound states need to be discerned. To detect complex formation at the single-molecule level, a number of fluorescence-based approaches have been developed. Conceptually the most straightforward way is to label the binding partners each with a fluorophore of different emission wavelengths. The observation of both fluorophores in the same point in space and time (colocalization) can be interpreted as the observation of a complex of ligand and binding partner [23].

Colocalization was used by Wang *et al.* to study the binding kinetics of the bacteriophage lambda repressor CI with its various operator sites on DNA [24••]. As done in many such single-molecule binding studies, the authors immobilized one component — the DNA — to a surface and imaged the frequent association and dissociation of fluorescently labeled binding partner — the repressor. The use of Total Internal Reflection Fluorescence (TIRF) imaging in this type of studies restricts the illuminated volume to just roughly 100 nm above the surface and helps minimize the fluorescence background [25]. Further, the ability to image a large surface area onto a CCD camera allows for the study of many binding reactions in parallel and thus greatly improves statistics. The association of a fluorescently tagged repressor protein from solution with dye-labeled, immobilized DNA was observed as a colocalization of the fluorescence from the DNA with that of the differently labeled protein. The use of DNA target molecules with different operator sequences and different lengths allowed for a characterization of the relative contributions of specific and nonspecific DNA binding to the overall binding kinetics [24••].

This colocalization method is limited, however, by the diffraction-limited nature of the optical elements in a fluorescence microscope. As a result of the inability to spatially separate two point sources closer together than the diffraction limit (typically a few 100 nm), it is impossible to determine with certainty whether two binding partners are physically associated or merely in proximity. An approach that is substantially more sensitive to true association uses Fluorescence Resonance Energy Transfer (FRET), a process in which the excitation energy of a donor fluorophore is transferred to an acceptor dye via an induced dipole–dipole interaction. The strong distance dependence of this interaction causes the transfer efficiency to be close to unity in the case of a physical association and close to zero when the two binding partners are separated by more than 10 nm [26].

The strength of such a FRET-based approach is clear in the work of Karymov *et al.*, in which formation of synaptic DNA complexes was studied [27•]. The authors immobilized an unlabeled SfiI endonuclease to a microscope coverslip and visualized how fluorescently labeled DNA fragments associated with the protein. Binding of two DNA sequences to the protein, a requirement for cleavage activity, was demonstrated by the visualization of FRET between the two duplex molecules. Similar FRET-based approaches have been used to study the kinetics of association of proteins with SNARE complexes [28,29] and the interaction of chromatin remodeling factors with nucleosomes [30]. The obvious advantage of FRET-based methods is that they also allow the study of conformational changes within complexes [4,11,26,31–33].

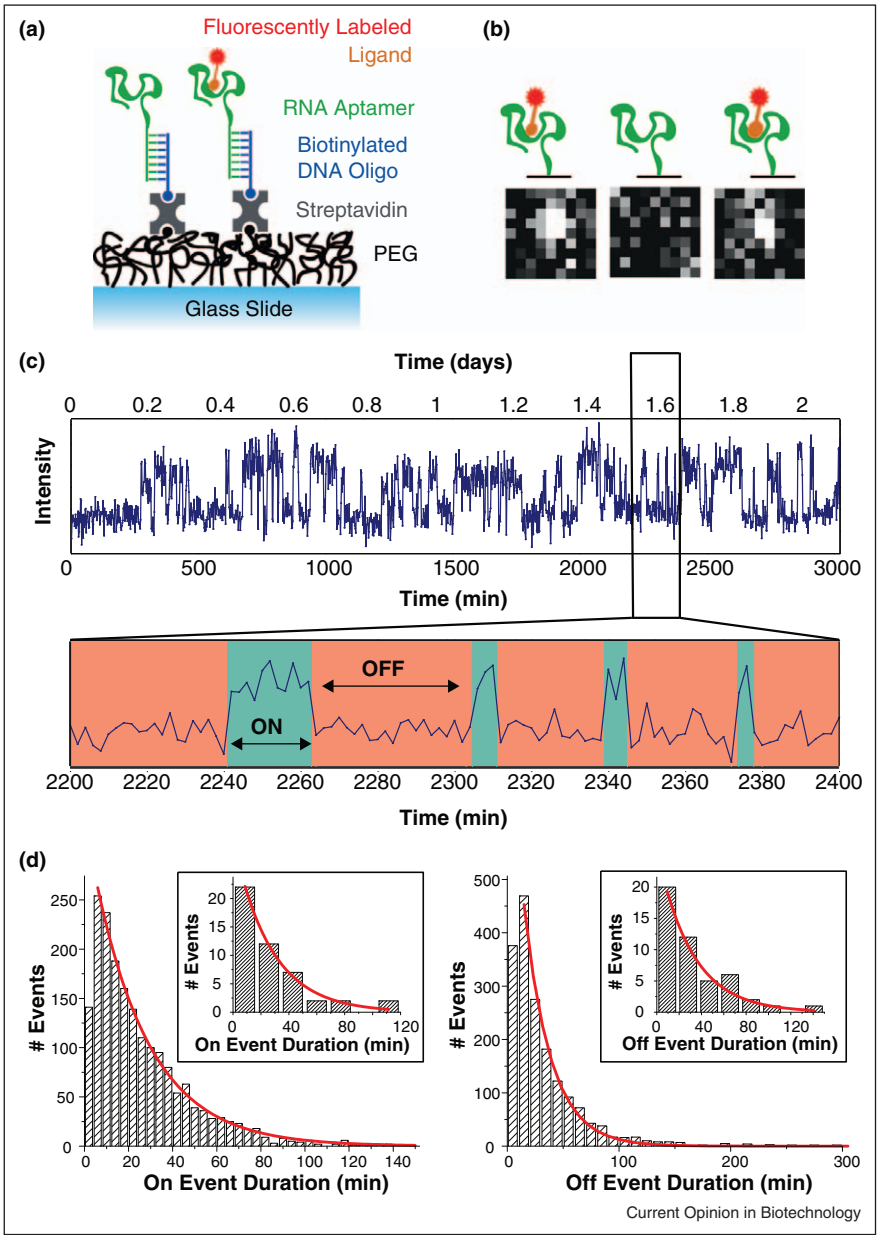
In the examples mentioned above, binding kinetics display characteristic timescales of seconds to minutes. For a diffusion limited (10^8 M^{-1} s^{-1}) bimolecular interaction, a very tight K_d of 10 nM translates to an off rate of 1 s^{-1}, resulting in binding lifetimes that match very well with the timescales accessible by CCD-based single-molecule fluorescence imaging. Many bimolecular interactions of interest, however, display nondiffusion limited association rates or slower dissociation rates, resulting in binding lifetimes that are much longer. Elenko *et al.* studied the kinetics of interaction between an RNA aptamer and its ligand, an interaction that is many orders of magnitude slower than diffusion limited [34••,35]. Specifically, the authors visualized the binding kinetics of a fluorescently labeled GTP ligand to a surface-immobilized RNA aptamer by detecting repeated immobilization events of labeled ligands (Figure 1). From the fluorescence 'on' times, the dissociation rate constant could be determined, whereas the 'off' times provided information on the association rate constant. Correction of mechanical drift and reduction of photobleaching by a noncontinuous illumination allowed these experiments to take place over many days, so that the slow binding kinetics of the aptamers could be studied [35].

To study fast binding kinetics (on the timescale of a millisecond and less), solution-based approaches have been developed that are based on measuring the fluorescence intensity fluctuations of small numbers of labeled molecules traversing a focused laser beam [36,37]. Colocalization [38] and FRET-based [39] strategies can be used in combination with this fluorescence correlation spectroscopy (FCS) method to obtain accurate information on the kinetic and thermodynamic properties of supramolecular complex formation or ligand binding [40•].

The concentration problem

Single-molecule fluorescence experiments are only possible in systems in which the concentration of fluorescent species is low enough to have at most one fluorescent molecule present per diffraction-limited volume and thus gives rise to a sufficiently low level of background fluorescence. Traditionally, background fluorescence reduction has been achieved by reducing the volume of sample that is illuminated, effectively lowering the number of fluorescing molecules present in the probed volume [41–44]. For example, TIRF microscopy confines laser excitation to a 100-nm thin layer above a glass surface, resulting in a drastic improvement of signal-to-background ratios of single-molecule images [25]. Nevertheless, the highest tolerable concentration at which individual fluorescent molecules can be imaged is in the order of 10 nM. At higher concentrations, more than one fluorescent molecule will be present per diffraction-limited volume and thus the background signal

Figure 1



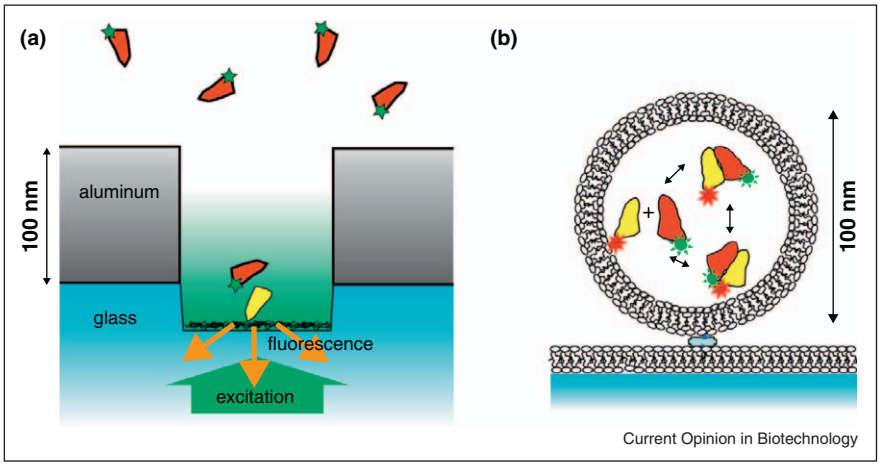
Single-molecule visualization of ligand-aptamer binding kinetics by Elenko *et al.* [34••] **(a)** Schematic depiction of aptamer immobilization on a functionalized surface. **(b)** Example of fluorescence images visualizing binding of a fluorescently labeled GTP to aptamer (left), dissociation (middle) and rebinding of another labeled ligand (right). **(c)** Fluorescence intensity trajectory of the particle depicted in panel (b). On and off events are denoted by green and red, respectively. **(d)** Histograms of all on and off events detected in the trajectories of a large number of single-aptamer traces. Insets show event distributions from the single trajectory shown in panel (c). Figure adapted with permission from [34••].

becomes many times larger than the signal of one single molecule.

In the case of thermodynamic equilibrium binding constants much higher than 10 nM, this concentration limit represents a significant technical hurdle to study the kinetics of bimolecular interactions. Even though rate constants of simple, noncooperative interactions can be extrapolated from kinetic information obtained at low concentrations, it is difficult to obtain a full kinetic picture from experiments at low concentrations in the case of more complicated, cooperative binding processes [45].

The most straightforward strategy to reduce fluorescent background while maintaining high concentrations is to fluorescently label only a portion of the probed molecules.

Figure 2



Strategies to reduce reaction volumes and thus allowing higher concentrations of labeled species in single-molecule binding experiments. **(a)** A hole with a size smaller than the wavelength of light is drilled into a glass-supported metal coating and illuminated by laser light. Such a zero-mode waveguide is too small to allow propagation of excitation light upward into the sample. Instead, illumination is restricted to the zeptoliter-sized (10^{-21} l) hole. This geometry allows detection of labeled molecules against the bulk solution background [48,49•,50••]. **(b)** Trapping of fluorescently labeled proteins in a surface-tethered, lipid nanovesicle. The confinement results in a high effective concentration that allows the observation of weak binding interactions [51–53,54•]. Panel (b) is reproduced with permission from [51].

Even though the fluorescence 'on' times — corresponding to the off-rate constant — will be unaffected by such an approach, the fluorescence 'off' times (providing information on the bimolecular association rate constant) will be reduced by the ratio of the unlabeled to labeled population. As a result, any heterogeneity in association kinetics will be invisible.

An exciting new direction that addresses the challenge of single-molecule detection at high concentrations of labeled species is the development of optical techniques that further reduce the illuminated volume in fluorescence microscopy [46]. Such methods will not only increase the resolution limit in fluorescence microscopy, with obvious impact in many fields related to imaging, but also significantly lower the number of emitting fluorophores contributing to background signal and allow single-molecule experiments at higher concentrations [47].

Another strategy is the reduction of the volume that contains the reactants. Early work in this direction revolved around the fabrication of microfluidic reaction chambers whose dimensions are significantly smaller than the resolution limit of optical microscopes (Figure 2a) [48]. A recent study by Miyake *et al.* demonstrated the use of these so-called zero-mode waveguides to observe the single-molecule binding kinetics of GroEL chaperonin with GroES cochaperonin at a concentration of 0.5 μM labeled cochaperonin (see Figure 2) [49•]. The earliest work using zero-mode waveguides led to the visualization of the incorporation of individual, fluorescently labeled nucleotides into a growing DNA chain by a single DNA polymerase [48]. This technique has been commercialized as a single-molecule DNA-sequencing platform and serves perhaps as the best example so far of the industrial application of single-molecule binding studies [50••].

Following a similar reasoning, researchers have been able to capture fluorescently labeled binding partners in small (100–200 nm), surface-tethered lipid nanovesicles and study their interactions through single-molecule FRET measurements (Figure 2b) [51–53,54•]. The volume of a 100-nm vesicle is two orders of magnitude smaller than that of a diffraction-limited volume and thus allows proteins to be visualized at the single-molecule level at much higher effective concentrations than hitherto possible.

Conclusion

The single-molecule techniques reviewed here have started to become important tools in understanding the interactions between biomolecules. These developments only represent the beginning of an exciting era in which single-molecule tools will play an increasingly important role in unraveling complex biochemical pathways. The majority of single-molecule studies thus far have focused on simple systems in idealized environments. However, cellular processes are typically not mediated by interactions between a single pair of biomolecules, but rather by large networks of interactions between many components in complex environments. Therefore, the utilization of single-molecule techniques to unravel the association and orchestration of the many components

required for the functioning of large macromolecular assemblies is an important future direction. Also, bringing these single-molecule techniques to the study of kinetics of molecular interactions inside living cells will increase our quantitative understanding of intermolecular interactions in the complex and crowded intracellular environment.

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